

CONCISE COMMUNICATION

Pathogenesis of Human and Bovine *Cryptosporidium parvum* in Gnotobiotic Pigs

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To compare the pathogenesis of human genotype 1 (HuG1) and bovine genotype 2 (BoG2) *Cryptosporidium parvum*, neonatal gnotobiotic pigs were given 1–10 HuG1 or BoG2 oocysts. The prepatent and patent periods were significantly longer for HuG1 than for BoG2 *C. parvum* (prepatent, 8.6 vs. 5.6 days; patent, 16.6 vs. 10.3 days). BoG2-infected pigs developed significantly more severe disease than did HuG1-infected pigs. BoG2 parasites were seen microscopically throughout the intestines during the prepatent and patent periods. HuG1 parasites were only detected during the patent period in the ileum and colon but colonized the mucosal surface in significantly larger numbers than did BoG2. Moderate-to-severe villus/mucosal attenuation with lymphoid hyperplasia was seen throughout the intestines of BoG2-infected pigs, whereas lesions in HuG1-infected pigs were mild to moderate and restricted to the ileum and colon. These findings provide additional support for the hypothesis that human and bovine *C. parvum* genotypes may be separate species.

Cryptosporidium parvum causes acute infectious diarrhea in humans and animals worldwide. Molecular studies have identified at least 2 major genotypes within human-pathogenic *C. parvum*: human genotype 1 (HuG1) and bovine genotype 2 (BoG2). These genotypes have biological differences in terms of host range; BoG2 isolates appear to be infectious for a wide variety of mammalian species, whereas HuG1 isolates appear to be highly selective, infecting principally humans [1]. This difference is important, because most human cases of cryptosporidiosis (>75%) are caused by HuG1; <25% are caused by BoG2 [1, 2].

The biological behavior of BoG2 *C. parvum* has been studied in several animal models, including the gnotobiotic pig [3–7], and can be defined by time to onset of oocyst shedding, or prepatent period; duration of shedding, or patent period; and severity of disease and pathologic lesions. The prepatent periods of BoG2 isolates in calves [5], mice [6], pigs [3], and humans [7] vary considerably. BoG2-infected calves and gnotobiotic pigs typically show symptoms of moderate-to-severe diarrhea

with weight loss and sometimes death due to dehydration and wasting. The biological behavior of HuG1 *C. parvum* in animals has not been studied until now because of the parasite's inability to infect nonhuman hosts. However, longitudinal studies in humans suggest that the human and bovine genotypes behave differently in the human host [8, 9].

High doses (thousands to millions of oocysts) of BoG2 *C. parvum* are traditionally used in animal pathogenesis studies, although most naturally acquired *Cryptosporidium* infections result from ingestion of relatively few oocysts. In fact, the risk of *C. parvum* infection is heightened as a result of the parasite's low infective dose. The recent finding that HuG1 isolates can infect neonatal gnotobiotic pigs [3, 4] has made possible comparative studies on the biological behavior of HuG1 and BoG2 *C. parvum* in a single host species. We believe that the present study is the first to compare the clinical and pathologic outcome of HuG1 and BoG2 *C. parvum* infections by using small numbers of oocysts (1–10 oocysts) to better emulate naturally acquired infections.

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Methods

BoG2 *C. parvum* GCH1 was supplied to our laboratory by S. Tzipori (Tufts University, School of Veterinary Medicine, Grafton, MA). GCH1 was first isolated from a patient with AIDS and was subsequently maintained by passage in neonatal calves. HuG1 *C. parvum* H2132 and H2265 were obtained from persons infected in waterborne disease outbreaks in South Carolina and Nebraska, respectively. Each *C. parvum* isolate was serially passaged in gnotobiotic pigs and confirmed to be BoG2 or HuG1 before and after

selected pig passages by nested polymerase chain reaction restriction fragment-length polymorphism (RFLP) targeting the small subunit rRNA gene [10].

For the first pig passage, day-old gnotobiotic pigs were fed 10^3 and 10^5 oocysts extracted from infected calf or human feces by immunomagnetic beads (IMB) (Dynabeads Anti-*Cryptosporidium* [no. 730.01]; Dynal). Pigs were euthanized 24–48 h after the detection of oocyst shedding by acid-fast stain and UV light microscopy [11], and their intestinal contents were collected and stored at 4°C. For subsequent passages, oocysts were extracted from pig intestinal contents by IMB and counted by hemocytometer. Sterile water was added to create a suspension of 10^3 oocysts/mL, and a 5- μ L droplet of each oocyst suspension was then placed on a drop slide and examined microscopically ($\times 400$ magnification). Droplets with 1–10 oocysts were covered with 50 μ L of 1% low-melting-point agarose (Life Technologies) and fed to pigs within 1 h. In the present study, 1–10 oocysts from the intestinal contents of the third (for strain H2132) and fifth (for strains GCH1 and H2265) pig passages were isolated as described and fed to pigs.

Forty gnotobiotic pigs were derived and maintained as described elsewhere [12]. At age 1 day, 15 pigs were fed 1–10 HuG1 oocysts, 15 pigs were fed 1–10 BoG2 oocysts, and 10 pigs were fed agarose. Pigs were monitored daily for clinical disease and oocyst shedding by acid-fast stain and UV light microscopy [11]. Feces were examined and scored as follows: 0, normal; 1, pasty; 2, opaque liquid; and 3, watery. Diarrhea was considered to be present at scores ≥ 2 . Dehydration was described as mild (visible vertebrae), moderate (prominent vertebrae and eyes retracted in orbit < 1 mm), and severe

(prominent protruding vertebral and pelvic bones and eyes retracted in orbit > 1 mm).

Three pigs from each infected group and 2 uninfected control pigs were killed 3 days after inoculation (DAI) and 0, 7, 21, and 42 days after onset (DAO) of oocyst shedding. Intestinal contents from each pig were collected and stored at 4°C. Sections of duodenum, jejunum, ileum, and colon were collected as described elsewhere [13], placed in fixative (Prefer; Anatech) for 24–48 h, processed through graded alcohols, embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin-eosin (HE).

Slides were coded and examined by an examiner who had no knowledge of infecting genotype or strain. The average intestinal infection rate per 100 linear micrometers in 10 random fields per intestinal section was evaluated at $\times 400$ magnification and scored as follows: 0, no parasites detected; 1, 1–10 parasites/100 μ m; 2, 11–30 parasites/100 μ m; and 3, > 30 parasites/100 μ m. Villus height and width, crypt height and width, and epithelial cytoplasmic vacuolation at villus tips were used to determine the extent of mucosal attenuation and were scored as follows: 0, same as in control pigs; 1, mild; 2, moderate; and 3, severe. The extent to which lymphoid cells had infiltrated the submucosa and the lamina propria and the size of organized submucosal lymphoid follicles (i.e., Peyer patches) determined the extent of lymphoid hyperplasia and was scored as follows: 0, same as in control pigs; 1, mild; 2, moderate; and 3, marked.

Between-group differences in mean diarrhea scores, dehydration, and prepatent and patent periods were identified by the general linear model (SAS Institute). We used χ^2 analysis to compare data

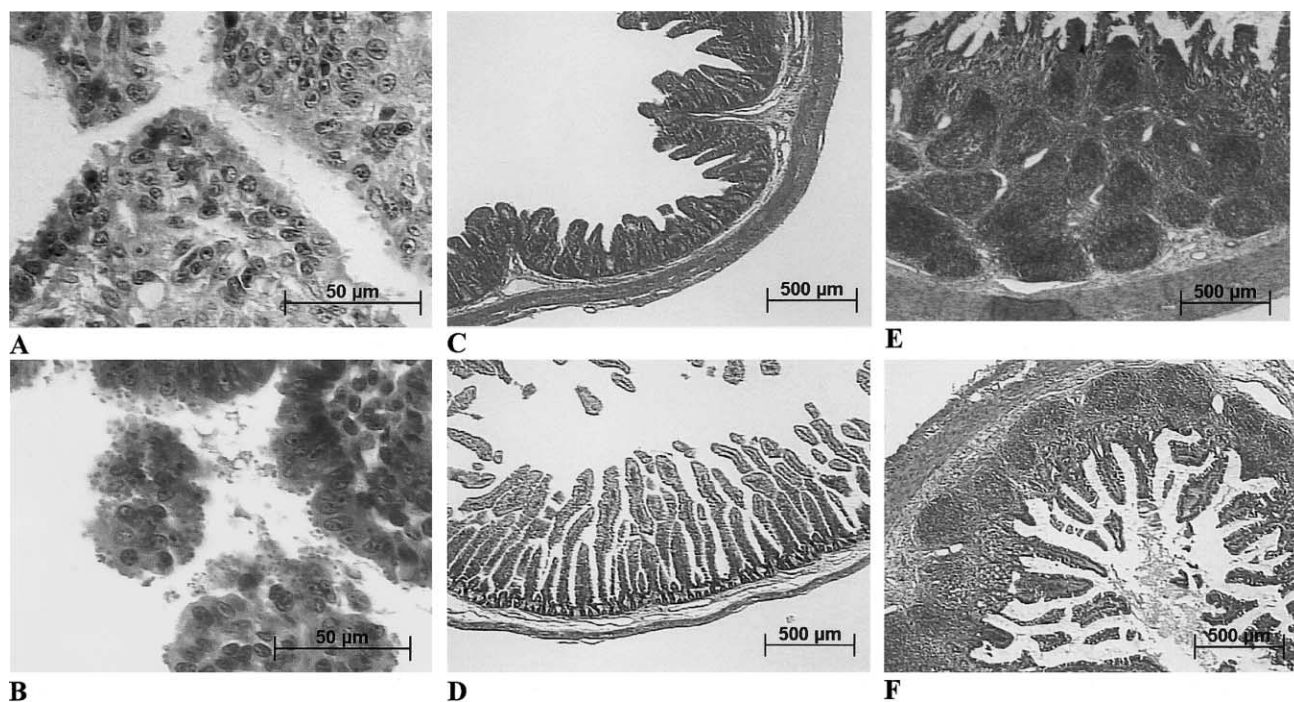


Figure 1. Photomicrographs show peak oocyst infection rates in ileum (A and B), villus attenuation in small intestine (C and D), and lymphoid hyperplasia in cecum (E and F) tissues from gnotobiotic pigs infected with bovine (A, C, and E) or human (B, D, and F) *Cryptosporidium parvum* (hematoxylin-eosin stain; original magnifications, $\times 400$ [A and B] and $\times 25$ [C–F]).

Table 1. Mean scores for pathologic lesions in the duodenum (D), jejunum (J), ileum (I), and colon (C) of gnotobiotic pigs infected with human and bovine *Cryptosporidium parvum*.

Genotype, time point	Parasite burden ^a				Lymphoid hyperplasia ^b				Villus attenuation ^c			
	D	J	I	C	D	J	I	C	D	J	I	C
Human												
3 Days after inoculation	0	0	0	0	0	0	0	0	0	0	0	0
Days after onset												
0	0	0	2.3	1.7	0	0	1	1	0	0	1	1
7	0	0	2	1.7	0	0	1	1	0	0	1	1
21	0	0	0	1	0	0	1.5	1	0.7	1	1	0.1
42	0	0	1	0	0	0	1.6	1	0	0	1	0
Bovine												
3 Days after infection	1	0	0	0	1	0	0.7	0	0.1	0	0	0
Days after onset												
0	1	0.3	0.3	1	1.7	1	1	1	1.1	1	0	1
7	1.3	0.7	1.3	1	1	1.5	1.5	1.5	1.1	1	1	0.5
21	0	0	1	0.3	1	1	1	2	1	0.5	1	2
42	0	0	0	0	1	1	1.3	1.3	0	0	0	0

NOTE. For each *C. parvum* genotype, 3 pigs were infected.^a Score: 0, no parasites detected; 1, 1–10 parasites/100 μ m; 2, 11–30 parasites/100 μ m; 3, >30 parasites/100 μ m.^b Score: 0, same as control pigs; 1, mild; 2, moderate; 3, marked.^c Score: 0, same as control pigs; 1, mild; 2, moderate; 3, severe.

for pathologic lesions between groups. $P \leq .05$ was considered to be significant.

Results

The prepatent period for HuG1-infected pigs was significantly longer (mean \pm SE, 8.6 ± 0.5 days) than for BoG2-infected pigs (5.6 ± 0.6 days) ($P = .001$). HuG1-infected pigs shed oocysts for 16.6 ± 1.8 days, whereas BoG2-infected pigs shed oocysts for 10.3 ± 1.7 days ($P = .03$). In the first week after the onset of shedding, HuG1-infected pigs had daily fecal scores of 1.8 ± 0.16 , and BoG2-infected pigs had significantly higher daily fecal scores of 2.6 ± 0.14 ($P = .009$). Daily fecal scores of control pigs were 0.48 ± 0.8 . During the patent period, BoG2-infected pigs developed moderate-to-severe dehydration and weight loss, whereas HuG1-infected pigs showed mild-to-no dehydration or weight loss in comparison with control pigs.

Microscopic examination of HE-stained tissue sections revealed BoG2 parasites in the duodenum, jejunum, ileum, and colon (figure 1A). BoG2 parasites were detected in the intestinal tissues of pigs killed during the prepatent (3 DAI) and patent (0–21 DAO) periods. However, HuG1 parasites were only seen during the patent period in the ileum and colon. No HuG1 parasites were detected in the duodenum and jejunum at any time during the study (table 1). Significantly more HuG1 parasites than BoG2 parasites were observed per villus in the ileum ($P = .05$) at 0 DAO (figure 1A and 1B).

Moderate lymphoid hyperplasia was seen at 0 DAO in the duodenum, at 7 DAO in the jejunum and ileum, and at 7 and 21 DAO in the colon of BoG2-infected pigs (table 1 and figure 1E). Other intestinal sections showed mild lymphoid hyperplasia and infiltration of scattered neutrophils. Lymphoid hyperplasia in the HuG1-infected pigs was noted only during the patent period. Mild lymphocytic infiltrates and rare inflam-

matory cells were seen at this time in the ileum and colon only (table 1 and figure 1F). The difference between the mean lymphoid hyperplasia scores for the 2 genotypes in the duodenum ($P = .002$), jejunum ($P = .02$), ileum ($P = .02$), and colon ($P = .008$) during the patent period was statistically significant.

Mild and moderate villus attenuation with epithelial sloughing was seen at 0 DAO and 7 DAO in the duodenum and at 21 DAO in the colon of BoG2-infected pigs (table 1). All other intestinal sections showed only mild villus attenuation between 3 DAI and 21 DAO. Comparatively few HuG1-infected pigs showed mild villus/mucosal attenuation during the patent period, and attenuation was restricted to the ileum and colon (0–21 DAO; figure 1C and 1D). The difference between the mean villus attenuation scores for the 2 genotypes in the duodenum ($P = .007$), jejunum ($P = .04$), and ileum ($P = .02$) during the patent period was statistically significant.

Discussion

Infection of day-old gnotobiotic pigs with ≤ 10 HuG1 or BoG2 oocysts resulted in extensive parasite colonization and disease. It has been suggested that serial passages in a host may increase virulence, with ingestion of lower doses achieving higher infection rates and more-severe disease in young gnotobiotic pigs [3]. Our findings suggest otherwise: the minimum infective dose of each isolate for gnotobiotic pigs was a single oocyst, after the first pig passage. In addition, the 8–9- and 5–6-day prepatent periods of HuG1 and BoG2 isolates in gnotobiotic pigs were evident after the first passage and remained so after several additional passages. The HuG1 isolates also elicited minimal disease in all subsequent passages, whereas the BoG2 isolate elicited moderate-to-severe disease accompanied by loss of weight that often was not regained. Examination of intestines from pigs in these early and subsequent serial pas-

sages revealed parasite infection rates, parasite tissue distribution, and pathologic lesions similar to those described in the present study (L.A.W., unpublished data).

Preliminary studies of 2 other BoG2 and 3 other HuG1 isolates in gnotobiotic pigs similarly have shown that all BoG2 strains have shorter prepatent periods (3–6 vs. 8–12 days) and have elicited more disease than HuG1 strains (L.A.W., unpublished data). Thus, the clinical outcome of a *C. parvum* infection in gnotobiotic pigs appears to be determined in part by the genotype of the parasite, and serial low-dose passage of the *C. parvum* used in this study did not significantly alter the strain virulence for gnotobiotic pigs.

HuG1-infected pigs had significantly more parasites in the ileum than did the BoG2-infected pigs, yet HuG1-associated intestinal lesions were only mild to moderate, compared with BoG2-associated lesions. This apparent discrepancy between infection intensity and lesion severity may be the result of unique parasite virulence factors yet to be identified. Differences in the host's immune response to each genotype may also contribute to the observed discrepancy.

We have demonstrated elsewhere that a strong time-dependent correlation exists between intestinal mucosal cytokine responses, disease onset, intestinal pathology, disease resolution, and parasite clearance in BoG2-infected pigs [3]. The immune response to BoG2 parasites may limit parasite load and eliminate infection sooner but simultaneously contribute to pathology and disease, whereas the absence of such a response would permit greater parasite replication for a longer period of time, with minimal pathology, as was observed in HuG1-infected pigs in the present study. Comparative studies of the gnotobiotic pig's cytokine and antibody responses to BoG2 and HuG1 *C. parvum* isolates are under way.

The observed differences between tissue colonization sites for HuG1 and BoG2 *C. parvum* also may help to explain the differences seen in the clinical outcome of infection in the same host species. BoG2 *C. parvum* colonized the entire small intestine (duodenum, jejunum, and ileum) and colon, whereas HuG1 *C. parvum* colonized only the ileum and colon. Because the upper small intestine is important in regulating absorption of nutrients and electrolytes, hindrance of its function often leads to a malabsorptive diarrhea. We noted a loss in epithelial integrity and villus attenuation of the small intestine, which was associated with the presence of parasites within the epithelial brush border and moderate-to-severe clinical disease in BoG2-infected pigs. Thus, we propose that the colonization site of the parasite within the host is a determinant of disease expression by that host. A clinical case study in patients with AIDS who had a history of cryptosporidiosis showed that different anatomic locations of the parasite produced different disease outcomes [14].

In conclusion, HuG1 and BoG2 *C. parvum* were identified by RFLP and used to infect gnotobiotic pigs. During sequential low-dose passages, these isolates showed distinctly different biological behaviors in the pig host, in terms of prepatent and patent periods, disease severity, pathology, and tissue colonization sites, that correlated with the strain's genotype. Given the increasingly significant molecular and biological differences between these genotypes that are being identified, reexamination and redefinition of the *Cryptosporidium* taxa may be in order.

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